

# Characteristics of T $\phi$ 3, a Bacteriophage for *Bacillus stearothermophilus*<sup>1</sup>

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A bacteriophage (T $\phi$ 3) which infects the thermophilic bacterium *Bacillus stearothermophilus* ATCC 8005 was isolated and characterized. Infection of the bacterium by the bacteriophage was carried out at 60 C, the optimal growth temperature of the host. At 60 C, the phage had a latent period of 18 min and a burst size of about 200. The phage was comparatively thermostable in broth. The half-life of T $\phi$ 3 was 400 min at 60 C, 120 min at 65 C, 40 min at 70 C, and 12 min at 75 C. The activation energy for the heat inactivation of T $\phi$ 3 was 56,000 cal. The buoyant density of T $\phi$ 3 in a cesium chloride density gradient was 1.526 g/ml. Electron micrographs of T $\phi$ 3 indicate that the phage has a head that is 57 m $\mu$  long. The dimensions and shape of the head are compatible with those of a regular icosahedron. Each edge of the head is 29 m $\mu$  long. The tail of T $\phi$ 3 is 125 m $\mu$  long and 10 m $\mu$  wide. There are about 30 cross-striations that are spaced at 3.9-m $\mu$  intervals along the tail. Under the conditions investigated, T $\phi$ 3 adsorbed slowly to the host. Only 2.8% of the phage adsorbed in 10 min at 60 C, the normal incubation temperature that was used. T $\phi$ 3 was not infective to four other thermophilic strains or to two mesophilic strains of bacteria.

There have been a number of reports dealing with bacteriophages for thermophilic bacteria (2, 8, 14, 15, 17, 20, 22, 23, 25, 28-31). These reports indicate that some of these phages possess rather unique characteristics. To study the structure of nucleic acids and its relationship to the structure of the proteins of simple thermostable organisms, we initiated a program for the systematic isolation and characterization of bacteriophage for the thermophilic bacterium *Bacillus stearothermophilus*.

The present report deals with the isolation and characteristics of a bacteriophage (T $\phi$ 3) for *B. stearothermophilus* ATCC 8005.

## MATERIALS AND METHODS

**Host organism.** The host bacterium was selected as a single-step mutant of *B. stearothermophilus* ATCC 8005 resistant to 1 mg per ml of streptomycin sulfate that was included in agar plates. This host is designated *B. stearothermophilus* ATCC 8005 S<sup>R</sup>.

**Media.** The TYNGC broth used for liquid bacterial and phage cultures and for dilution of phage suspension for assay purposes contained 10 g of tryptone (Difco), 5 g of yeast extract (Difco), 10 g of NaCl, and

1,000 ml of distilled water. Glucose (1 g) and CaCl<sub>2</sub> (to  $2 \times 10^{-3}$  M) were added aseptically after autoclaving. Agar plates contained 20 to 30 ml of TYNGC plus 2.5% agar (Difco). Top agar consisted of TYNGC plus 0.8% agar.

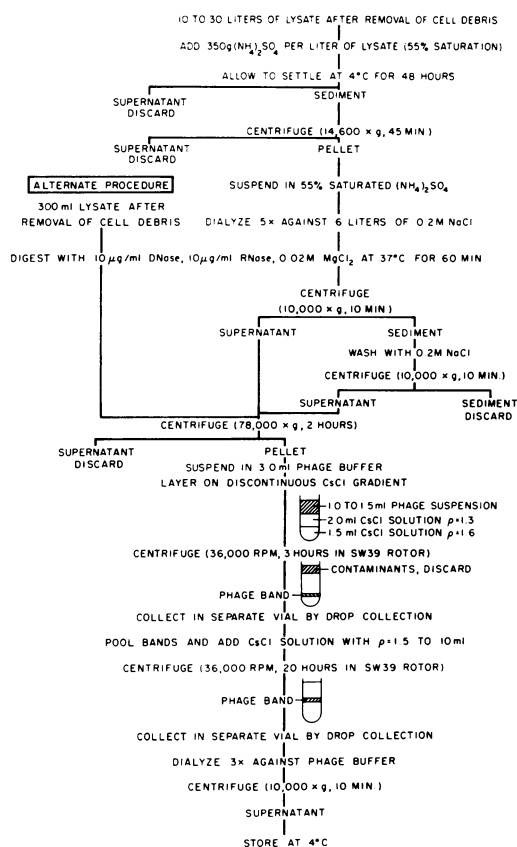
**Phage isolation.** Phage were isolated from soil samples by the method of Romig and Brodetsky (19), except that *B. stearothermophilus* ATCC 8005 S<sup>R</sup> was used as the host organism and the incubation temperature was 60 C. Three successive single-plaque isolations were carried out to insure homogeneity of the phage stock suspensions.

**Incubation conditions.** The incubation temperature for broth cultures and plates was 60 C unless otherwise noted. Small cultures (20 to 30 ml) were incubated in a 125-ml screw-capped flask in a G76 Gyrotory water bath shaker (New Brunswick Scientific Co., New Brunswick, N.J.). Large cultures (15 liters) were incubated in a 5-gal (about 19 liters) carboy in a heated water bath. These large cultures were aerated vigorously with heated filtered air.

Phage were assayed by use of the agar-layer technique (1). Unless otherwise noted, 0.1 ml of phage suspension was plated with 0.5 ml of a log-phase culture of *B. stearothermophilus* ATCC 8005 S<sup>R</sup> ( $5 \times 10^7$  cells per ml) as a lawn in 1.5 ml of top agar. Agar plates for phage assay were incubated for 5 to 6 hr at 60 C. The atmosphere in the incubator was humidified to prevent drying of the plates. The concentration of infective phage particles in a phage suspension, as determined by this method, is reported as plaque-forming units (PFU) per milliliter.

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FIG. 1. Purification scheme for bacteriophage T $\phi$ 3.

Phage lysates were produced by infecting a culture of the host organism, containing  $5 \times 10^6$  cells per ml, with  $5 \times 10^6$  to  $10^7$  PFU/ml. Incubation was continued until the bacteria in the culture lysed and the culture cleared (about 300 min). The cell debris in small cultures was removed by centrifugation at  $10,000 \times g$  for 10 min. For stock phage suspensions, the supernatant fluid was passed through a sterile HA membrane filter (Millipore Corp., Bedford, Mass.) and stored at 4 C. The cell debris in large cultures was removed by centrifugation in the KSB-R continuous-flow system (Ivan Sorvall, Inc., Norwalk, Conn.) at 16,500 rev/min and at a flow rate of 300 ml/min. Lysates contained  $2 \times 10^{10}$  to  $3 \times 10^{10}$  PFU/ml.

**Purification.** The procedure used for the purification of large quantities of T $\phi$ 3 is illustrated in Fig. 1. Deoxyribonuclease and ribonuclease were obtained from the Sigma Chemical Co., St. Louis, Mo. Phage buffer consisted of 0.2 M NaCl and 0.01 M tris (hydroxymethyl)aminomethane (pH 7.15) at 25 C. The discontinuous CsCl gradient used was similar to that of Trautman and Bresse (26). The drop collection technique was that of Weigle, Meselson, and Paigen (27). The CsCl used in the discontinuous gradients and in the phage buoyant-density experiments was designated 99.9% CsCl (Kaweccki Chemical Co., New

York, N.Y.). The precipitate developed by this product was filtered out before use. A filtered stock solution with a density of 1.51 g/ml had an optical density of 0.022 at a wavelength of 260 m $\mu$ . Optical-quality CsCl (Harshaw Chemical Co., Cleveland, Ohio) was used in all other experiments.

**Electron microscopy.** A diluted purified phage suspension was dialyzed against three changes of distilled water and then mixed (1:1) with either 4% phosphotungstic acid (5), pH 7.0, in 0.4% sucrose solution or a saturated solution of uranyl acetate (3, 9). The mixture was then placed on a standard electron-microscope grid that was coated with a collodion film reinforced with a thin layer of evaporated carbon. The excess solution was removed with the edge of a piece of filter paper. The preparation was then air-dried before examination in a Phillips EM200 electron microscope.

**Determination of the buoyant density of T $\phi$ 3.** The buoyant density of T $\phi$ 3 in a cesium chloride density gradient (6) was determined as follows. A purified suspension of phage T $\phi$ 3 and a purified suspension of  $\lambda^+$  were diluted and mixed with a CsCl solution whose density was 1.51 g/ml. The final density of the solution was 1.50 g/ml. Centrifuge tubes for the SW39 rotor of a Spinco (model L) ultracentrifuge were filled with 3.3 ml of mixed phage suspension, and this suspension was overlaid with paraffin oil. The mixed phage suspension was then centrifuged in the SW39 rotor for 20 hr at 37,000 rev/min at 4 C in the ultracentrifuge. The rotor was allowed to slow to a stop without a brake. The tubes were carefully removed and the contents were fractionated by the drop-collecting technique (27). The individual drops were collected into 2.0 ml of sterile TYNGC broth, except that every 10th drop was collected in an empty screw-cap vial, for density determination. The screw-cap vials were capped immediately after a drop was collected. The

FIG. 2. Plaques produced by T $\phi$ 3. Plates were incubated for 5 hr at 60 C.

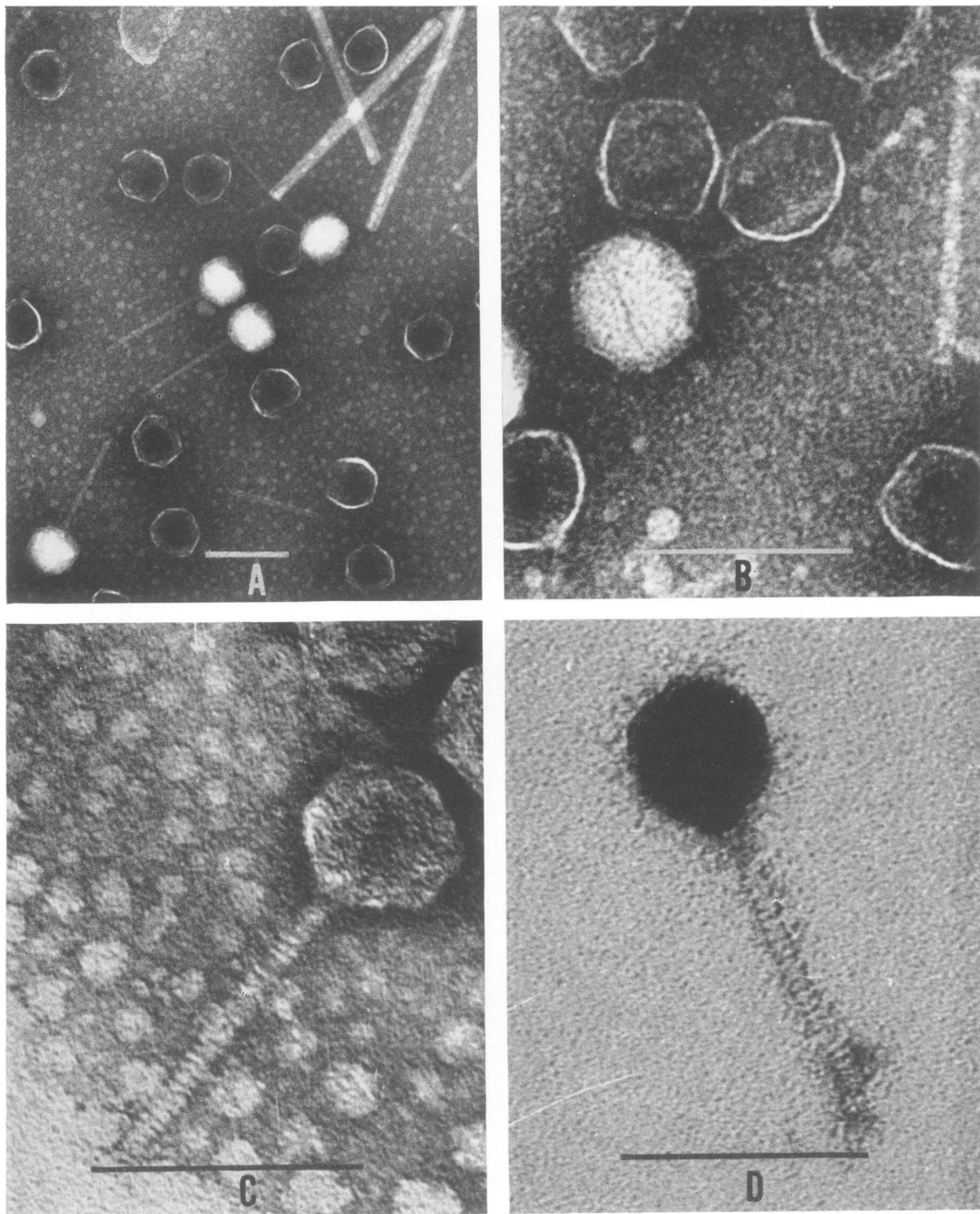


FIG. 3. Electron micrographs of bacteriophage  $T\phi 3$ . (A) Phosphotungstic acid negative stain with tobacco mosaic virus as an internal length standard. (B) Phosphotungstic acid negative stain. (C) Uranyl acetate negative stain. (D) Uranyl acetate positive stain. The solid bar in each micrograph represents  $0.1 \mu$ .

refractive index of each fraction in the screw-capped vials was measured with a Zeiss refractometer. The density ( $\rho^{25}$ ) of these fractions was determined from the relationship established by Ifft, Voet, and Vinograd (10). The slope of the graph of  $\rho^{25}$  versus fraction num-

ber was then determined by the method of least squares by use of density data from every tenth fraction from fraction number 20 through fraction number 80. The actual density gradient was adjusted so that the density at the  $\lambda^+$  peak (see below) was  $1.508 \text{ g/ml}$  (13).

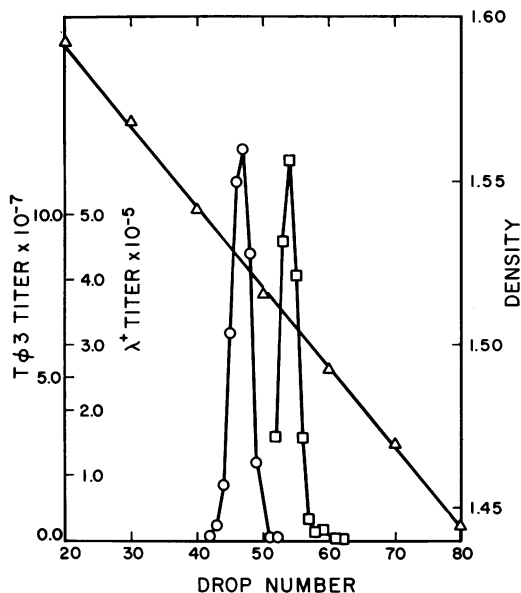


FIG. 4. Buoyant density of bacteriophage T $\phi$ 3. Purified samples of T $\phi$ 3 and  $\lambda^+$  in a solution of CsCl whose density was 1.50 were centrifuged at 37,000 rev/min in the SW39 rotor. After centrifugation, one-drop fractions were collected in 2.0 ml of TYNGC broth, except that every 10th drop was collected in a screw-cap vial. The refractive index of each 10th drop was determined and the density was calculated from the refractive index. The density gradient was determined and the density scale was adjusted so that the density at the  $\lambda^+$  peak was 1.508. The other fractions were assayed for T $\phi$ 3 and  $\lambda^+$ , with *Bacillus stearothermophilus* 8005 S<sup>R</sup> or *Escherichia coli* C600, respectively, as indicators. (O) T $\phi$ 3 titer; (□)  $\lambda^+$  titer; (Δ) density.

The position of the T $\phi$ 3 phage band was determined by assaying each fraction for T $\phi$ 3. The position of the  $\lambda^+$  band was determined by assaying each fraction for  $\lambda^+$ , using *Escherichia coli* C600 as a lawn. Plates for the  $\lambda^+$  assay were incubated overnight at 35 C. Wild-type  $\lambda$  phage and the culture of *E. coli* C600 were the gift of E. T. Young.

**Thermal stability of T $\phi$ 3.** The stability of T $\phi$ 3 at high temperatures was examined by placing 20 ml of phage suspension containing  $2 \times 10^8$  to  $4 \times 10^8$  PFU/ml in a 125-ml screw-cap flask in a water bath at the appropriate temperature. The temperature was allowed to equilibrate for 5 min. Samples (1 ml) were taken at appropriate times, cooled in an ice bath, and assayed for T $\phi$ 3. The natural logarithm of the ratio of the initial titer to the titer at time  $t$  was plotted against the time  $t$ . A straight line was fitted by least squares to the data at each temperature, and the time of one-half survival (half-life) was determined from the slope.

## RESULTS

**Isolation.** Phage were isolated from 4 of the 11 soil samples examined. One of these four soil samples contained three types of phages, as dis-

tinguished by the morphology of the plaques produced by them. One of the three phages from this sample produced large clear plaques and was capable of producing a high-titer lysate. This phage was chosen for further study and was called T $\phi$ 3 (thermophilic phage 3).

Plaques produced by T $\phi$ 3 on TYNGC medium are illustrated in Fig. 2. The size of the plaques varies from pin points up to about 2 mm. This variation in plaque size could be eliminated by increasing the initial concentration of bacteria in the lawn by a factor of 10.

Attempts to infect four other thermophilic bacterial strains (*B. stearothermophilus* ATCC strains 7953, 7954, 10149, and 12016) and two mesophilic strains (*E. coli* C and C600) with T $\phi$ 3 were unsuccessful.

**Phage morphology.** Electron micrographs of T $\phi$ 3 are illustrated in Fig. 3. Tobacco mosaic virus (TMV) was included in some preparations as an internal length standard. The length of TMV was assumed to be 298 m $\mu$  (32). The length of the tail of T $\phi$ 3 was found to be 125 m $\mu$ . This length did not vary under the various staining conditions used and was used as a standard for magnification calibration when TMV was not present.

The length of the regular hexagon-shaped head, as determined from preparations that were stained

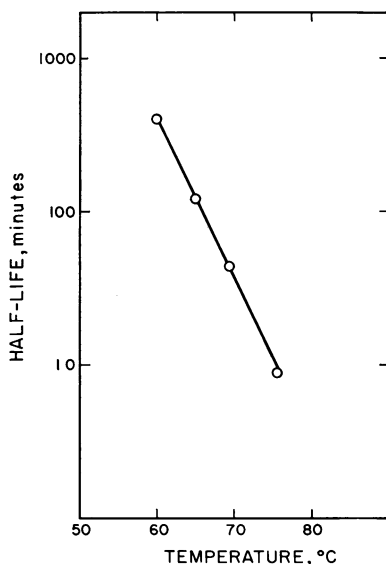


FIG. 5. Thermal stability of T $\phi$ 3. A 20-ml sample of T $\phi$ 3 in TYNGC broth was placed in a water bath at the appropriate temperature. After temperature equilibration, 1-ml samples were taken at appropriate times and assayed for T $\phi$ 3. The time of one-half survival (half-life) was determined from the slope of a plot of the natural logarithm of the ratio of the initial titer to the titer at time  $t$  versus the time  $t$ . This half-life thus determined is here plotted against temperature.

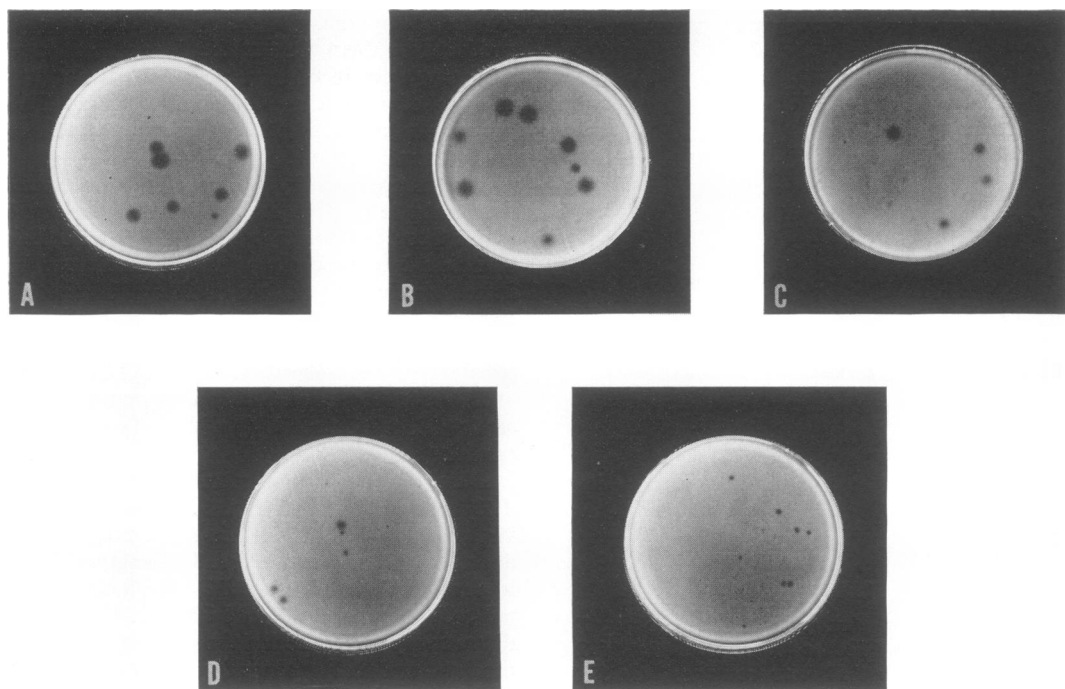


FIG. 6. Continued increase in plaque size at various temperatures. Plates that had been incubated at 60 C for 6 hr (full plaque development) were incubated for 8 hr at the indicated temperature. (A) 60 C; (B) 48 C; (C) 37 C; (D) 25 C; (E) 4 C.

negatively with uranyl acetate (Fig. 3C), is 57 m $\mu$ . The length of each side of the head is 29 m $\mu$ . The tail is 10 m $\mu$  wide, and there are about 30 cross-striations that are spaced at 3.9-m $\mu$  intervals along the tail. The cross-striation pattern changes near the junction of the tail and head. There may also be small tail fibers at the end of the tail (Fig. 3D).

**Buoyant density of T $\phi$ 3.** The buoyant density of phage T $\phi$ 3 was determined from the location of the infective phage particles in a cesium chloride density gradient. Bacteriophage T $\phi$ 3 yielded a single sharp band at a density of 1.526 g/ml. Three determinations all gave this value for the buoyant density of T $\phi$ 3. The results of a density gradient experiment are illustrated in Fig. 4.

When large quantities of phage were purified by the density gradient technique, a small second band sometimes occurred at a density of 1.48 g/ml. The significance of this band was not determined.

**Growth characteristics.** The latent period of T $\phi$ 3 at 60 C in TYNGC medium was found to be 18 min. Single-burst experiments (1) in which unadsorbed phage were removed by centrifugation indicated that the burst size of T $\phi$ 3 is about 200. With a bacterial concentration of  $2.5 \times 10^7$  bacteria per ml, only 2.8% (as determined from the number of infected bacteria in the single-burst

experiments) of the phage adsorbed to the bacteria in 10 min. The phage adsorption rate was independent of phage concentration. If calcium ion was not included in the medium, the plaques formed by T $\phi$ 3 were small and the efficiency of plating was low.

**Thermal stability.** The thermal stability characteristics of phage T $\phi$ 3 are illustrated in Fig. 5. In broth, the half-life of T $\phi$ 3 at 60 C is 400 min. At higher temperatures, the phage is less stable; the half-life is 120 min at 65 C, 40 min at 70 C, and 12 min at 75 C. The activation energy ( $\Delta H_a$ ) for the heat inactivation of T $\phi$ 3 in broth in the temperature range 60 to 75 C is 56,000 cal.

**Phage lytic enzyme.** The possible existence of a phage enzyme that attacks and breaks down the bacterial cell wall (21) was investigated by continuing the incubation of plates containing phage T $\phi$ 3 on a bacterial lawn for longer than 6 hr; the plaque size continued to grow as a turbid halo formed around the clear central plaque (Fig. 6). This halo was produced rapidly (4 hr) at 60 C, slowly (8 hr) at 48 C, and even more slowly at 35 and 25 C. At 4 C, no halo was produced. There was essentially no further bacterial growth after the 6-hr initial incubation, as judged by the turbidity of the areas of bacterial growth. One phage was found in each of two samples, out of a total

of 10 samples that were taken from the outer edges of three halos. The remaining eight samples contained no phage particles. Samples taken from the centers of the plaques contained approximately  $10^6$  PFU.

#### DISCUSSION

The investigation reported here has dealt with the isolation of a thermophilic bacteriophage and the determination of some of the characteristics of this phage. This investigation was a necessary preliminary to a more detailed study, now in progress, of the protein and nucleic acid of the phage.

Several problems arose during this study. One of these was the variability in plaque size if special precautions were not taken. This variability is apparently due to a low rate of adsorption of T $\phi$ 3 to the host. This is indicated by two observations, the low adsorption rate of T $\phi$ 3 to the host in broth and the uniformity of the plaque size brought about by an increased concentration of the host on the plates. It is not known whether the low adsorption rate is due to a scarcity of specific adsorption sites on the surface of the bacterial cell or to a low affinity of the phage for the adsorption sites under the culture conditions used. The more pronounced variability in plaque morphology that was observed when  $\text{Ca}^{++}$  was not included in the medium indicates that  $\text{Ca}^{++}$  or another divalent cation is required for either adsorption or growth. The direct effect of low  $\text{Ca}^{++}$  concentration upon adsorption in broth was not determined.

In the study of the morphology of T $\phi$ 3, it was observed that the phosphotungstic acid negative-staining procedure produced many incomplete particles. As many as 9 of 10 particles in some preparations were incomplete. In most of the preparations, the phage heads appeared to be swollen.

In contrast to this, all of the phage in the uranyl acetate negative-stained preparations were intact and the heads were usually symmetrical. In positive-stained preparations, the outer edges of the protein coat were poorly resolved. For these reasons, the measurements for the head dimensions were taken from negative-stained preparations.

The general dimensions and shape of the head are compatible with those of a regular icosahedron. However, the resolution of capsomeres has not been high enough, and double-shadowing experiments have not been performed to give a definitive answer to this problem.

The cross-striation pattern along the tail indicates that the tail may have a stacked-ring structure, with one or several subunits per striation. A helical arrangement of individual subunits, such as that found in T2 (6) and T5 (12), cannot be

ruled out by this striation pattern, even though the striations were observed to be perpendicular to the long axis of the tail.

As would be expected, T $\phi$ 3 is more thermostable than are phages of mesophiles. In broth, for example, the half-life of the *E. coli* phage T1 is about 12 min at 65 C (18). Another *E. coli* phage, T7, is less stable, having a half-life of less than 1 min at 60 C. The half-life of T $\phi$ 3 at 65 and 60 C is 120 and 400 min, respectively. At 65 C, T $\phi$ 3 is four times as stable as the thermophilic phage TP-1 (28).

The high  $\Delta H_a$  for heat inactivation of T $\phi$ 3 indicates that protein denaturation is primarily responsible for this inactivation (1). This evidence, taken together with the evidence for the relative thermostability of T $\phi$ 3, indicates that the phage proteins may have rather unique structural characteristics. These characteristics might consist of a unique amino acid composition, as in the case of the  $\alpha$ -amylase of *B. stearothermophilus* (7), or of a folding arrangement of the polypeptides that is particularly resistant to thermal denaturation. A further study of the proteins of T $\phi$ 3 could yield some insight into the relationship between the primary amino acid sequence and the tertiary structures of proteins in general.

A logical candidate for further study of thermostable phage proteins is the lytic enzyme responsible for lysis of the host by T $\phi$ 3. A detailed study of this enzyme would be very meaningful in view of the current research on the genetic control of the structure of T4 phage lysozyme (24) and on the detailed structure of egg-white lysozyme (4, 11). At this time, it is not known how closely T $\phi$ 3 lytic enzyme resembles a true lysozyme in its mode of enzymatic activity.

A subsequent report will deal with the deoxyribonucleic acid of T $\phi$ 3.

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